

Predominant Th2/Tc2 Polarity of Tumor-Infiltrating Lymphocytes in Human Cervical Cancer¹

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Cytotoxic T lymphocytes (Tc) play a central role in cellular immunity against cancers. The cytotoxic potential of freshly isolated tumor-infiltrating lymphocytes (TILs) is usually not expressed. This suggests the possible existence of as yet unspecified and perhaps complex immunosuppressive factors or cytokines that affect the anti-tumor capacity of these TILs in the tumor milieu. In the present study, we demonstrated for the first time that TILs derived from human cervical cancer tissue consist mainly of Th2/Tc2 phenotypes. In vitro kinetic assays further revealed that cancer cells could direct the tumor-encountered T cells toward the Th2/Tc2 polarity. Cancer cells promote the production of IL-4 and down-regulate the production of IFN- γ in cancer-encountered T cells. The regulatory effects of cervical cancer cells are mediated mainly by IL-10, and TGF- β plays only a synergistic role. The cancer-derived effects can be reversed by neutralizing anti-IL-10 and anti-TGF- β Abs. IL-10 and TGF- β are present in cancer tissue and weakly expressed in precancerous tissue, but not in normal cervical epithelial cells. Our study strongly suggests important regulatory roles of IL-10 and TGF- β in cancer-mediated immunosuppression. *The Journal of Immunology*, 2001, 167: 2972–2978.

Since the discovery of polarized cytokine patterns among CD4⁺ T cells, studies have concentrated on the physiologic significance of the polarized T cells in immunity (1–6). The polarized T cell phenotype was originally based on the analyses of mouse T cell clones cultured in vitro, with dichotomous and cross-regulatory patterns of cytokine produced by two functionally distinct subsets, Th1 and Th2 cells (2–6). Recently, it has been demonstrated that naive CD8⁺ T cells are able to differentiate into a T cytotoxic-1 (Tc1)³ subset that produces IL-2 and IFN- γ , or a Tc2 subset that produces IL-4, IL-5, and IL-10 (5–9). In human T cell clones, Th1/Tc1 cells preferentially develop during infections with intracellular bacteria and enhance phagocyte-mediated immunity, whereas Th2/Tc2 cells predominate during metazoan parasite infestations and in atopic diseases (4–6). Although both types of CD8⁺ T cell subsets are thought to possess a cytolytic function in vitro, there is substantial evidence indicating that CD8⁺ T cells secreting type 1 and type 2 cytokines in vivo might have great relevance to immune responses (10–13). In a recent study of TCR-transgenic mice, Tc2 has been demonstrated to be less protective than Tc1 against pulmonary influenza virus

infection (13). Because Tc cells play a central role in cellular immunity against cancers, alterations of Tc1/Tc2 subsets in the tumor microenvironment may change host anti-tumor immune responses (8–12).

In human cervical cancer (CC) as well as in other cancers, depressed anti-tumor immunity of Tc cells has been observed (14–16). The cytotoxic potential of freshly isolated tumor-infiltrating lymphocytes (TILs) is usually not expressed (16–18). This suggests the possible existence of as yet undefined mechanisms that affect the anti-tumor capacity of these TILs in the tumor milieu. Previously, we have demonstrated that cancer cells may alter the functional composition of anti-tumor effector cells (e.g., CD3⁺CD25⁺ T cells) within the tumor milieu (19, 20). We have further illustrated that a decreased proportion of CD4⁺ T cells is responsible for the reversed CD4/CD8 ratio of TILs in human CC (20). Because the tumor-reactive CD4⁺ T cells can be activated through the MHC class II pathway to produce lymphokines and amplify the cytotoxic activity of CD8⁺ T cells (21, 22), a decreased proportion of CD4⁺ T cells with a reversed CD4/CD8 ratio in vivo may result from functional alterations of T cells within the tumor microenvironment.

In this study, we examined the functional characteristics of TILs by investigating their cytokine expression (23, 24). We used an in vitro mixed autologous lymphocyte and tumor-cell coculture (MLTC) model to evaluate the kinetic expression of cytokines by cancer-encountered T cells (25). Our results demonstrate for the first time that activated T cells in the human cancer milieu predominantly express a Th2/Tc2 phenotype, and that cancer cells can directly drive the tumor-encountered T cells toward Th2/Tc2 polarity through an IL-10- and TGF- β -mediated pathway.

Materials and Methods

Immunocyte isolation and activation for cytokine production

Patients with stage Ib CC admitted for operation were prospectively enrolled in this study. A complete history was obtained for each patient. Informed consent was obtained for collecting the materials in this study.

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³ Abbreviations used in this paper: Tc, T cytotoxic (cell); TIL, tumor-infiltrating lymphocyte; CC, cervical cancer; MLTC, mixed autologous lymphocyte and tumor-cell coculture; MLNC, mixed autologous lymphocytes and noncancerous epithelial cells coculture; T+C, T cell and cancer cell coculture.

After radical hysterectomy, the surgical specimens were carefully examined by experienced pathologists. Tissue specimens were excised aseptically immediately after operation from at least four different tumor sites and two sites of normal cervical epithelium of the same tumor patients. Fragments of tissue were washed with PBS for removal of contaminated blood. CC cells, cervical epithelial cells, and TILs were isolated according to methods described previously (19, 20, 25). For matched comparison, venous blood of each patient was obtained before operation and transferred to test tubes containing heparin. PBMCs were isolated by Ficoll-Hypaque (1.077 density) and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium. The recovered cells were checked and counted for viability with the trypan blue staining method. Purified TILs and PBMCs (1×10^6 cells/ml) were activated in medium containing PMA at 25 ng/ml, ionomycin at 1 μ g/ml, and brefeldin A at 10 μ g/ml. The activated cells were incubated in a 37°C, 5% CO₂ humidified incubator for 18 h. The activation status was monitored by surface staining of CD69.

Surface and intracellular staining

Monoclonal Abs labeled with FITC, PE, and PerCP were used for triple-color flow cytometry as previously described (19, 20, 25). For surface Ag staining, 20 μ l anti-CD4-FITC, anti-CD8-FITC, and/or anti-CD3-PerCP (BD Immunocytometry Systems, San Jose, CA) were used for every set of 1×10^6 activated T cells. After incubation for 30 min, cells were washed twice with 1 ml lysing buffer, and fixed with 1 ml fixation buffer containing 1% paraformaldehyde. Cells were washed twice with 500 μ l permeabilization buffer containing 0.5% saponin, and stained with 20 μ l fluorescence-conjugated anti-cytokine mAbs for an additional 30 min at room temperature. Anti-IFN- γ -PE, anti-IL-5-PE (BD PharMingen; San Diego, CA), and anti-IL-4-FITC plus anti-IFN- γ -PE (BD Immunocytometry Systems) were used for intracellular staining. Anti-mouse cytosol-IgG1-FITC + cytosol-IgG2a-PE was used as negative control. Triple-color flow cytometry was performed on a FACSCalibur (BD Biosciences, Mountain View, CA). Data were acquired with CellQuest software (BD Biosciences) by the use of forward-scatter/side-scatter thresholds with 10,000 gated events. We used Leukogate to measure the proportion of lymphocytes in the sample being studied. In triple-color assays with cytokine expression, data were further gated on FL3⁺ cells with a CD3 trigger for evaluation of T cell subsets.

Purification of CD8⁺ cytotoxic T lymphocytes

CD8⁺ T cells were isolated from TILs by use of an indirect magnetic labeling system with a MACS (Miltenyi Biotec, Gladbach, Germany). The cells were suspended in PBS medium containing 20 μ l of Hepten-Ab Cocktail (containing CD4, CD11b, CD16, CD19, CD36, and CD56 Abs; Miltenyi Biotec) and incubated in a cold room (6–12°C) for 10 min. After repeated washing, 20 μ l of MACS anti-hepten microbeads per 10⁷ total cells was added. The suspension was further incubated for 15 min at 6–12°C, then washed carefully by the addition of 10–20 \times the labeling volume of the washing buffer. The magnetically labeled cells were passed through a MACS separator in a magnetic field. The column was rinsed with an adequate volume of buffer and repeatedly eluted. The effluent was collected as negative fraction, representing the enriched CD8⁺ T cell fraction. The cell purity was checked by flow cytometry after labeling with anti-CD8-FITC.

RT-PCR analysis of mRNA expression

We measured the cytokine mRNA expression in purified CD8⁺ T cells derived from TILs and PBMCs, CC cells, and normal cervical epithelial cells by RT-PCR. The mRNAs of cytokines involved in the functional T cell development were examined. Total cellular RNA was extracted and reverse-transcribed (0.1–0.5 mg RNA) in the presence of IL-4, IL-10, IL-12, IFN- γ , TNF- α , and TGF- β oligonucleotide primers (CLONTECH Laboratories, Palo Alto, CA). Human β -actin primers were used as positive controls. The PCR was performed in a DNA thermal cycler (GeneAmp DNA thermal cycler 480; PerkinElmer, Norwalk, CT) for 36 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 1 min, followed by polymerization at 72°C for 1 min. PCR-amplified products were separated on an agarose gel and visualized by staining with ethidium bromide.

Mixed lymphocyte and tumor cell coculture assay

To address the possible role of cancer cells in T cell polarity, we cultured PBMCs with autologous cancer cells or normal cervical epithelial cells (25). The procedures outlined were performed in duplicate in 12 mm \times 75 mm capped polystyrene test tubes. PBMCs were activated with 10 μ g/ml of PHA or anti-CD3 (1 μ g/ml) plus anti-CD28 (1 μ g/ml) and goat anti-mouse cross-linkers (2 μ g/ml; Immunotech, Marseille, France) in a 37°C,

5% CO₂ humidified incubator. A constant number (5×10^5) of PBMCs were cultured with autologous cancer cells (1×10^6) in the MLTC group. In the control group of mixed autologous lymphocytes and noncancerous epithelial cells coculture (MLNC), 5×10^5 activated T cells were cultured with 1×10^6 autologous normal cervical epithelial cells. The supernatant on days 1, 3, and 5 of coculture was collected at the same intervals and checked for the presence of IL-4 and IFN- γ by a standard ELISA. The cytokine expressions of T cells in MLTC and MLNC were measured by an intracellular staining method and flow cytometry as described in the previous sections.

ELISA for IL-4 and IFN- γ measurement

Supernatants of MLTC and MLNC were collected in small aliquots. The levels of IL-4 and IFN- γ were measured with a commercial ELISA kit (Endogen, Woburn, MA). A spectrophotometer set to 450–550 nm was used for measuring the OD of each well of the 96-well polystyrene microtiter plate. Triplicate readings were averaged and expressed in units of picograms per milliliter.

Expression of IL-10 and TGF- β in CC tissue

An avidin-biotin-peroxidase complex immunohistochemical staining method was performed for examination of the expression pattern of IL-10 and TGF- β in formalin-fixed and paraffin-embedded tissue sections. The anti-IL-10 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-TGF- β Ab was obtained from Serotec (Kidlington, Oxford, U.K.). The specificities of both Abs were provided by the manufacturers.

Effects of anti-IL-10 and anti-TGF- β Abs on T cell polarity

To clarify the functional role of cancer-derived IL-10 and TGF- β on T cell polarity, we added anti-IL-10 (BD PharMingen) and anti-TGF- β Abs (Serotec) to the previously established MLTC. Cancer cells (1×10^6) were first cultured in 1 ml of culture medium containing 10 μ g/ml of anti-IL-10 and/or anti-TGF- β Abs for 2 h and then cocultured with activated T cells (5×10^5 cells) in the T cell and cancer cell coculture (T+C) group. For matched comparison, a constant number (5×10^5) of activated T cells was cultured as the T-only group. Mouse anti-human IgG1 and rat anti-human IgG (10 μ g/ml) were used as isotype Ab controls. The supernatant of the coculture was collected at the same intervals and checked for the presence of IL-4 and IFN- γ .

Direct effects of recombinant IL-10 and TGF- β on T cell polarity

We further directly explored the functional role of IL-10 and TGF- β on T cell polarity. CD3⁺ T cells were purified by MACS and activated with anti-CD3 (1 μ g/ml) plus anti-CD28 (1 μ g/ml) and goat anti-mouse cross-linkers (2 μ g/ml) (Immunotech) as previously mentioned. The activated cells were labeled with anti-CD69-PE and checked by flow cytometry. Recombinant IL-10 and TGF- β (PeproTech, Rocky Hill, NJ) were added to the cultures of activated T cells (5×10^5) at a final concentration of 5 and 1 ng/ml, respectively. For matched comparison, a constant number (5×10^5) of activated T cells was cultured as the T-only group. In addition to the previously established MLTC (T+C group), anti-IL-10 and anti-TGF- β Abs were added in another set of MLTC (T+C plus anti-IL-10/anti-TGF- β group). The cytokine expression of gated CD3⁺ T cells was measured on day 3 by the intracellular staining method and flow cytometry as described in the previous sections. The supernatant of the coculture was collected at the same time and checked for the presence of IL-4 and IFN- γ . Studies were performed in triplicate.

Statistical analysis of data

Data analysis was performed with a statistical analysis system (R.6.12; SAS Institute, Cary, NC). The Wilcoxon signed-ranks test was used for comparison of the median expression ratio of cytokine expression in CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cell subsets between TILs and PBMCs. Kinetic data of MLTC and MLNC were analyzed by a generalized estimation equation for correlated data of repeated measurements and were expressed as mean \pm SD. Statistical significance was defined by a *p* value < 0.05.

Results

Determination of the subtypes of Tc cells in TILs

Histologic examination of the specimens revealed that all CC cases were squamous cell carcinomas. The viability of immunocytes was ~90–95% at the completion of the isolation procedure in all cases.

In triple-color flow cytometric assays with dual-cytokine intracellular staining of eight CC cases, gated CD3⁺ T cells (Fig. 1, *a* and *b*) derived from TILs expressed mainly IL-4, but not IFN- γ (Fig. 1*c*). In contrast, prominent expression of IFN- γ , but not IL-4, was observed in activated CD3⁺ PBMCs (Fig. 1*d*). Subsequent staining of dual-surface phenotyping in combination with cytokine intracellular staining showed that the expression of these cytokines was restricted to particular subsets of T cells (Fig. 1, *e-h*). Prominent expression of IL-5 with low expression of IFN- γ was demonstrated in both gated CD3⁺CD4⁺ (data not shown) and CD3⁺CD8⁺ TIL subsets (Fig. 1, *e* and *f*) derived from CC tissue.

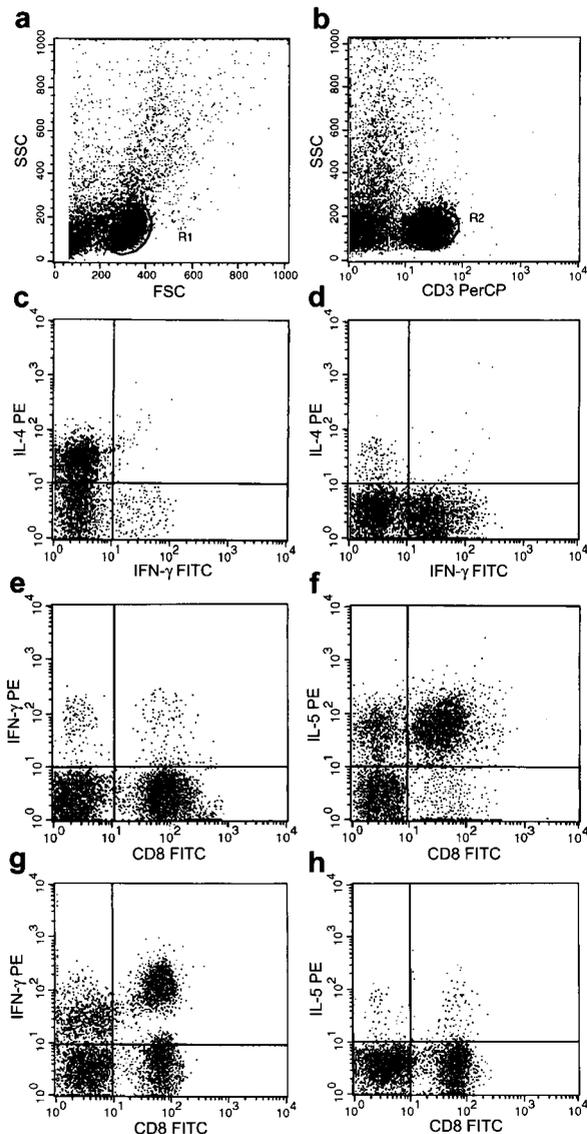


FIGURE 1. Representative dot-plots of dual-intracellular cytokine staining-gated CD3⁺ T cells in TILs and PBMCs. *a*, Data were acquired with forward scatter (FSC)/side scatter (SSC) thresholds with 10,000 gated events. We used leukogate (R1) to measure the proportion of lymphocytes in the sample being studied. *b*, In triple color assays with cytokine expression, data were further gated on FL3⁺ cells with a CD3 trigger (R2) for evaluation of the CD3⁺ T cell subsets. *c*, Prominent expression of IL-4 in gated CD3⁺ TILs derived from CC tissue. *d*, Prominent expression of IFN- γ in gated CD3⁺ PBMCs. *e*, Low expression of IFN- γ in gated CD3⁺CD8⁺ TILs derived from CC tissue. *f*, Prominent expression of IL-5 in gated CD3⁺CD8⁺ TILs derived from CC tissue. *g*, Prominent expression of IFN- γ in gated CD3⁺CD8⁺ PBMCs. *h*, Low expression of IL-5 in gated CD3⁺CD8⁺ PBMCs.

Contrarily, prominent expression of IFN- γ with low expression of IL-5 was detected in both activated CD3⁺CD4⁺ (data not shown) and CD3⁺CD8⁺ PBMCs (Fig. 1, *g* and *h*). Flow cytometric analyses further defined the ratios of intracellular cytokine expression in individual T cell subsets (Fig. 2, *a* and *b*). The median expression ratio of IL-5 on CD3⁺CD4⁺ cells was significantly higher in TILs than in PBMCs (60.71 vs 0.25%, $n = 8$, $p < 0.01$). A similar finding was obtained with CD3⁺CD8⁺ cells (66.73 vs 0.28%, $n = 8$, $p < 0.01$), which signified the predominant Th2/Tc2 patterns of cytokine expression in TILs.

We also analyzed the IL-4 and IFN- γ mRNA expression in CD8⁺ T cells isolated from TILs and activated PBMCs (Fig. 2*c*). Similar to the result obtained with intracellular staining, we observed the abundant expression of IL-4 mRNA, but low expression of IFN- γ mRNA, in CD8⁺ TILs. The PBMC-derived CD8⁺ T

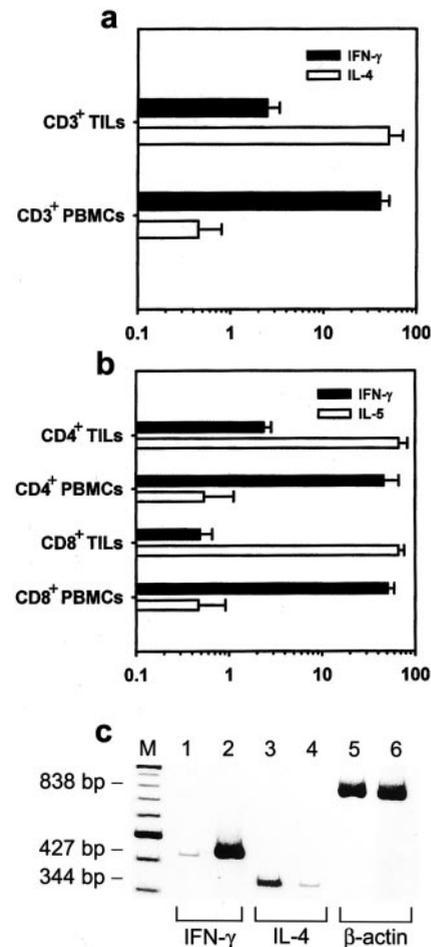


FIGURE 2. Polarized cytokine production in TILs and PBMCs after PMA and ionomycin activation. The data are representative of median expression ratios (%) of eight independent assays. *a*, IL-4 was predominantly expressed in the CD3⁺ TILs, with IFN- γ predominantly expressed in the corresponding CD3⁺ PBMCs. *b*, IL-5 was predominantly expressed in both the CD3⁺CD4⁺ and CD3⁺CD8⁺ TILs, with IFN- γ predominantly expressed in the corresponding CD3⁺CD4⁺ and CD3⁺CD8⁺ PBMCs. *c*, Analysis of IL-4 and IFN- γ mRNA expression in purified CD8⁺ T cells. The CD8⁺ T cells were isolated from TILs and PBMCs. For each sample, constant amounts of reverse-transcribed RNA were amplified. Human β -actin mRNA (838 bp) was used as positive control. Representative data show that prominent IL-4 mRNA (344 bp) was expressed in CD8⁺ TILs, but only weakly expressed in activated CD8⁺ T cells derived from PBMCs. IFN- γ mRNA (427 bp) was abundantly expressed in PBMC-derived CD8⁺ T cells, but weakly in TIL-derived CD8⁺ T cells (*lanes 1, 3*, and *5*, TILs; *lanes 2, 4*, and *6*, stimulated PBMCs; M, markers).

cells prominently expressed IFN- γ mRNA and low expression of IL-4 mRNA. The results emphasized that tumor-encountered T cells in the cancer milieu have a predominant Th2/Tc2 phenotype of mRNA expression and cytokine production.

Determination of the effects of cancer cells on TILs

It is possible that CC cells can direct the differentiation of activated T cells toward Th2/Tc2 polarity. To clarify this possibility, we used a previously established in vitro cancer-host interaction model and analyzed the cytokine production of IL-4 and IFN- γ in the supernatants of MLTC and MLNC (25). In six sets of repeated experiments, the mean concentration of IFN- γ in MLNC supernatants was 201.2 ± 16.4 pg/ml and increased >1- and 2-fold by days 3 and 5, respectively. However, the mean concentration of IFN- γ in the supernatants of MLTC was significantly lower than that of MLNC in day 5 cocultures ($n = 6, p < 0.01$) (Fig. 3a). The mean concentration of IL-4 in MLNC supernatants was 64.1 ± 6.6 pg/ml and increased by <1-fold by days 3 and 5. However, the mean concentration of IL-4 in the supernatants of MLTC was significantly higher than that in MLNC in day 3 and day 5 cocultures (increased >3- and 4-fold, respectively) ($n = 6, p < 0.001$) (Fig. 3b). It became evident that CC cells could promote the production of IL-4 and possibly abrogate the production of IFN- γ in cancer-encountered T cells.

Determination of immunosuppressive mediators expressed by cancer cells

To examine the possible cancer-derived immunosuppressive factors or cytokines that regulate the functional development or polarization of cancer-encountered T cells, we further compared the mRNA expression of immunoregulatory cytokines, including IL-4, IL-10, IL-12, IFN- γ , TNF- α , and TGF- β , in 30 samples of CC cells. IL-4, IL-12, IFN- γ , and TNF- α mRNAs were not detected in either cancer or epithelial cells. However, TGF- β and IL-10 mRNAs were highly expressed in the CC cells, but not in the normal cervical epithelial cells (Fig. 3c). We then studied the existence of TGF- β and IL-10 in CC by immunohistochemical staining of CC tissues from 47 patients. TGF- β and IL-10 were abundantly expressed in most, if not all cancer cells, but were not or only very weakly expressed in normal cervical epithelial or stromal cells (Fig. 4, a and b). The expression of TGF- β and IL-10 became obvious in transitional sections from normal cervical epithelium to carcinoma-in-situ and invasive cancer areas (Fig. 4, c and d). Unlike the expression of TGF- β and IL-10 in cancer cells, the expression of other types of immunoregulatory cytokines was not detected.

Determination of the function of IL-10 and TGF- β in T cell polarity

To characterize the possible role of IL-10 and TGF- β in the polarity of cancer-encountered T cells further, we directly incubated anti-IL-10 and anti-TGF- β Abs in the autologous MLTC. Using the kinetic experiments on MLTC described above, we determined whether specific blocking Abs could reverse or limit the cancer-induced effects. PBMCs were fully activated as matched controls (activated T-cells-only group). In six sets of matched experiments after 5 days of coculture, the mean concentration of IFN- γ in the supernatants of MLTC was significantly lower than that in the T-cells-only group (287.05 ± 17.12 vs 811.40 ± 30.96 pg/ml, $n = 6, p < 0.01$) (Fig. 5a). The mean concentration of IL-4 in the MLTC group was ~3-fold that in the activated T-cells-only group (347.06 ± 13.61 vs 103.39 ± 18.34 pg/ml, $n = 6, p < 0.01$) (Fig. 5b).

By addition of anti-IL-10 Ab, an increase in the mean concentration IFN- γ in the supernatants of MLTC was observed in day 3

and day 5 cocultures (Fig. 5a). The mean concentration of IL-4 in the supernatants of MLTC was synchronously reduced on day 3 and day 5 cocultures (48 and 57% decrease, respectively) (Fig. 5b). The effect of anti-TGF- β Ab on cancer-induced IL-4 production was not as obvious as that of the anti-IL-10 Ab, with only 19 and 25% decreases in day 3 and day 5 cocultures, respectively. However, adding both anti-IL-10 and anti-TGF- β Abs in MLTC achieved a maximal reduction of IL-4 in the supernatants, being 65 and 70% reductions in day 3 and day 5 cocultures, which were close to the mean concentrations of IL-4 in the supernatants of the T-cells-only group (Fig. 5b). The effects of anti-IL-10 and anti-TGF- β Abs were also observed in the patterns of IFN- γ in the supernatants of MLTC (Fig. 5a). The isotype controls did not alter the production of IL-4 or IFN- γ in the MLTC (data not shown). Our data illustrated that cancer cells promote the production of IL-4 and abrogate the production of IFN- γ in cancer-encountered

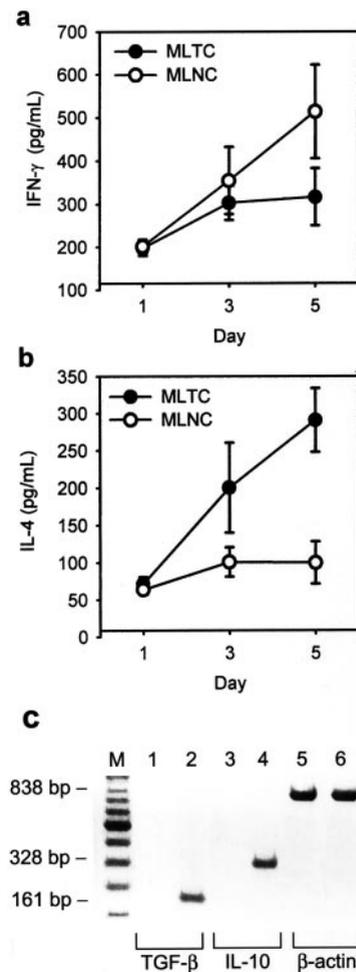


FIGURE 3. CC cells can drive activated T cells toward the Th2/Tc2 polarity. The levels of IL-4 and IFN- γ in the supernatants of MLTC (●) and MLNC (○) were analyzed by ELISA. *a*, The mean concentration of IFN- γ in the supernatants of MLTC was similar to that in MLNC in day 3, and lower than that of MLNC in day 5 cocultures ($p < 0.01$). *b*, The mean concentration of IL-4 in the supernatants of MLTC was significantly higher than that in MLNC in day 3 and day 5 cocultures ($p < 0.001$). Data from six independent experiments of cocultures in triplicate are presented as mean \pm SD. *c*, Analysis of TGF- β and IL-10 mRNA expression in normal cervical epithelial cells and CC cells. Human β -actin mRNA (838 bp) was analyzed as positive control. Representative data show that the TGF- β mRNA (161 bp) and IL-10 mRNA (328 bp) were highly expressed in CC cells, but not in epithelial cells (lanes 1, 3, and 5, normal epithelial cells; lanes 2, 4, and 6, CC cells; M, markers).

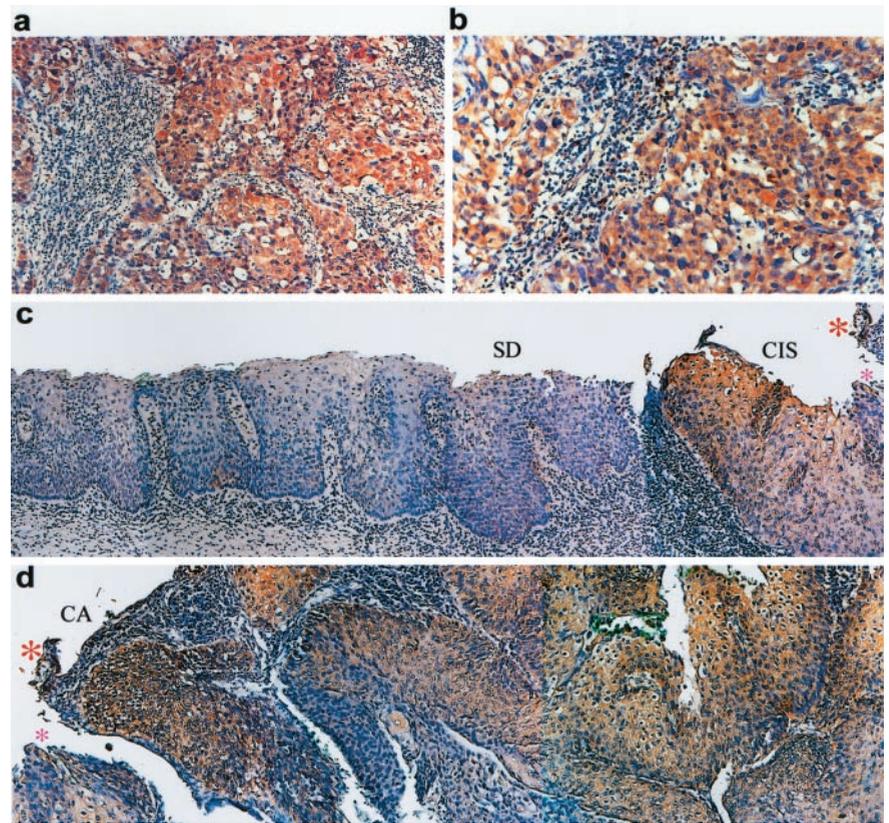


FIGURE 4. Expression of TGF- β and IL-10 in CC tissues. *a*, Abundant TGF- β expression in cancer cells. *b*, Abundant IL-10 expression in cancer cells. *c* and *d*, Abundant expression of IL-10 was noted in cancer cells, but not in normal cervical epithelial or stromal cells. CA, Cancer; CIS, carcinoma in situ; SD, severe-dysplasia epithelium.

T cells. These cancer-derived effects can be reversed by anti-IL-10 and anti-TGF- β Abs.

Compatible cancer-derived effects and recombinant IL-10/TGF- β on T cell polarity

To clarify the possible synergistic effect of cancer-derived IL-10 and TGF- β on the polarity of T cells, we further incubated activated T cells with recombinant IL-10 and TGF- β in addition to the established autologous MLTC. Using the kinetic experiments on MLTC and the intracellular staining technique described above, we directly determined the cancer-derived effects on cancer-encountered T cells. In eight sets of matched experiments after 3 days of coculture, the median expression ratio of IL-4 was significantly higher in the MLTC (T+C group) than in the activated T-cells-only group (77.28 vs 0.31%, $n = 8$, $p < 0.01$). The median expression ratio of IFN- γ in MLTC was lower than that in the T-cells-only group (0.46 vs 87.57%, $n = 8$, $p < 0.01$). The median expression ratio of IL-4 in T cells treated with recombinant IL-10 and TGF- β was consistent with that in MLTC (72.53 vs 77.28%). Adding both anti-IL-10 and anti-TGF- β Abs in MLTC significantly reduced the expression of IL-4 in CD3⁺ T cells (5.8 vs 77.28%, $n = 8$, $p < 0.01$). Similar results were seen in the measurements of IL-4 and IFN- γ of the coculture supernatants. The mean concentration of IL-4 in supernatants of the MLTC group was similar to that of culture of activated T cells in recombinant IL-10 and TGF- β (202.78 ± 74.51 and 199.29 ± 79.77 pg/ml, respectively). Anti-IL-10 and anti-TGF- β Abs in MLTC decreased the mean concentration of IL-4 to the levels of the T-cells-only group, being 56.97 ± 11.94 and 58.49 ± 8.78 pg/ml, respectively. It became evident that human cancer cells have well-matched effects as that of recombinant IL-10 and TGF- β on driving the polarity of cancer-encountered T cells. Importantly, adding anti-IL-10 and anti-TGF- β Abs can reverse the cancer-derived effects.

Discussion

Several lines of evidence have indicated that T cells exert regulatory effects on immune responses by secreting type 1 or 2 cytokines in vivo (10–13). These two types of cells may play different roles in both physiologic and pathologic conditions. Tc1 cells have been shown to induce a stronger delayed-type hypersensitivity reaction than do Tc2 cells in a murine disease model (11). Increases in Th2/Tc2 cell populations have been demonstrated in certain infections and human diseases (4–6). A type 1-to-type 2 cytokine shift may reflect a reduced protective cellular immunity against tumors (26), although both Tc1 and Tc2 subsets are thought to possess a cytolytic function in vitro. The cytolytic potential of Tc1 and Tc2 is proposed to be perforin-mediated. Tc1, but not Tc2, may involve Fas-mediated cytolysis (12).

In the present study, we directly demonstrated that the T cell (both CD4⁺ and CD8⁺ cell) polarity can be driven toward the Th2/Tc2 subset by IL-10 and TGF- β from human cancer cells. In triple-color flow cytometric assays with dual-cytokine intracellular staining, CD3⁺ T cells derived from TILs expressed mainly type 2 cytokine patterns. We further defined the ratios of intracellular cytokine expression in individual CD3⁺CD4⁺ and CD3⁺CD8⁺ T cell subsets in TILs and signified the predominant Th2/Tc2 patterns of cytokine expression in the cancer milieu. Using an in vitro coculture model of MLTC, we directly demonstrated that human cancer cells could promote the production of IL-4 and possibly abrogate the production of IFN- γ in cancer-encountered T cells through the synergistic effect of IL-10 and TGF- β .

Our findings link both in vivo data that, for the first time, reveal polarity in TILs and show in vitro for the first time that human cancer cells can drive encountered T cells toward Tc2 polarity by cancer-derived mediators. Polarization toward Th2/Tc2 subsets may signify a down-regulation of the cytotoxic potential of TILs

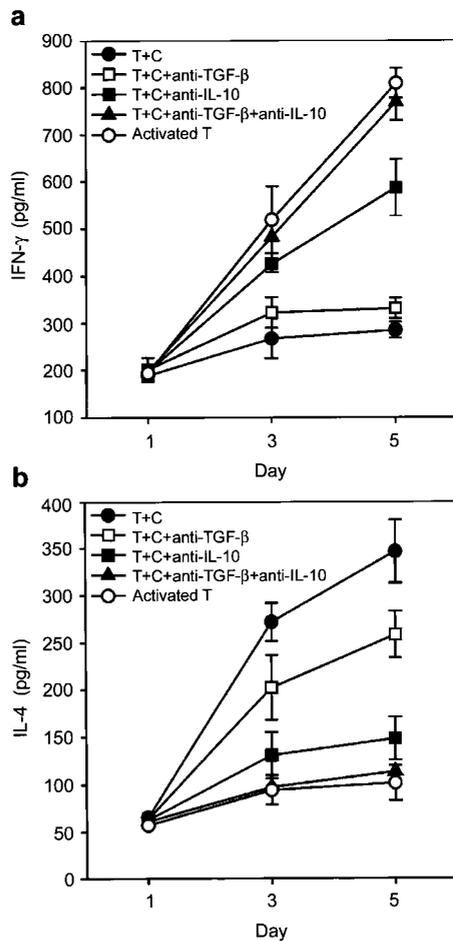


FIGURE 5. Effects of cancer-derived IL-10 and TGF- β on the polarity of cancer-encountered T cells. The levels of IL-4 and IFN- γ in the supernatants of MLTC were analyzed by ELISA. *a*, The mean concentration of IFN- γ in the supernatants of MLTC was significantly lower than that of the T-cells-only group after 5 days of coculture ($p < 0.01$). By adding anti-IL-10 and anti-TGF- β Abs, an increase in the mean concentration IFN- γ in the supernatants of MLTC was observed in day 3 and day 5 cocultures. *b*, After 5 days of coculture, the concentration of IL-4 in the MLTC group was ~3-fold that in the T-cells-only group ($p < 0.01$). By addition of anti-IL-10 Ab, the mean concentration of IL-4 in the supernatants of MLTC was reduced in day 3 and day 5 cocultures. Adding both anti-IL-10 and anti-TGF- β Abs to MLTC achieved a maximal reduction of IL-4 in the supernatants, close to the mean concentrations of IL-4 in the supernatants of the T-cells-only group. Data from six independent experiments of cocultures in triplicate are presented as mean \pm SD. Various combinations of Abs added to the MLTC cultures (T+C) (●) include anti-TGF- β (□), anti-IL-10 (■), and anti-IL-10 + anti-TGF- β (▲). Activated T cells (T cell-only) were used as controls (○).

and/or render the tumor cells resistant to TIL-mediated cytotoxicity. Our results confirm that cancer cells may attain immune escape by changing the effector composition of the host immune cells. We further explore the issues of proposed cancer-host immune interactions based on previous *in vivo* murine models and *in vitro* cell line results (27–40).

TGF- β and IL-10 are secreted spontaneously by a variety of human malignant tumors, including breast, colon, ovary, cutaneous basal/squamous cancers, metastatic melanoma, fibrosarcoma, and pancreatic carcinoma (27–35). The mechanisms responsible for the increased expression by tumor cells are not known. It has been suggested that tumor-derived IL-10 may be associated with the down-regulation of anti-tumor immunity by inhibiting the cytotoxicity of CTLs, tumor Ag presentation, and Th1 cell develop-

ment (27, 28, 35, 36). However, related studies in the literature showed indirect evidences of either elevated IL-4 and IL-10 mRNA, or mixed type 2 cytokine patterns in cancers (28, 37, 38). In the present study, we directly demonstrated predominant *in vivo* Th2/Tc2 patterns of cytokine expression in subsets of TILs, and stratified that tumor-derived IL-10 promotes a shift toward Th2/Tc2 polarity. The immunosuppressive role of IL-10 has been emphasized as a strategy used by tumors to escape immunosurveillance (39). A recent study by Steinbrink et al. (40), showing that IL-10-treated human dendritic cells can induce anergy in tumor-specific cytotoxic CD8⁺ T cells and result in failure to lyse tumor cells, further elucidates the down-regulatory role of IL-10 in tumor-mediated immunosuppression.

TGF- β has pleiotropic and potent immunosuppressive effects in the regulation of various physiologic processes. Tumor-derived TGF- β has been shown to down-regulate the generation of tumor-specific cytotoxic lymphocytes (41–43) and the secretion of immunoregulatory cytokines by T cells (42). TGF- β can be a strong costimulator that promotes a shift toward Th2 polarity in rat activated T cells (44). In a tumor-bearing murine model, TGF- β has been shown to shift the Th1/Th2 balance through a direct, IL-10-mediated pathway (45). By secreting IL-10 and TGF- β , tumor cells can affect the activation, proliferation, and differentiation of TILs in a synergistic manner. We have demonstrated in the MLTC model that human cancer cells can drive T cell polarity toward the Th2/Tc2 subsets by the synergistic effects of TGF- β and IL-10. The cancer-derived IL-10 and TGF- β may play a synergistic role in the induction of immune privilege in local cancer milieu (46). It is worthy of note that both IL-10 and TGF- β had expressions that progressed from carcinoma *in situ* to invasive cancer, but were not expressed in normal cervical epithelium of transitional sections (Fig. 4, *a–d*). The correlated presence of IL-10/TGF- β and Th2/Tc2 polarity of TILs in cancer tissue demonstrates that cancer-derived mediators can be active in modulating T cell functions.

In conclusion, we demonstrated that activated T cells in a human CC milieu predominantly expressed the Th2/Tc2 phenotype. We showed that cancer cells could drive the tumor-encountered T cells toward Th2/Tc2 polarity, which may be attributed to the prominent IL-10 and TGF- β expression in CC cells.

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